The role of glycerol in the pathogenic lifestyle of the rice blast fungus *Magnaporthe oryzae*.

Andrew J. Foster, Lauren S. Ryder, Michael J. Kershaw and Nicholas J. Talbot

School of Biosciences, University of Exeter, Stocker Road, Exeter EX4 4QD, United Kingdom

Correspondence: N.J.Talbot@exeter.ac.uk

Summary

The rice blast fungus *Magnaporthe oryzae* elaborates a specialised cell called an appressorium, which is used to breach the tough outer cuticle of a rice leaf, enabling the fungus entry to host plant cells. The appressorium generates enormous turgor by accumulating glycerol to very high concentrations within the cell. Glycerol accumulation and melanisation of the appressorium cell wall collectively drive turgor-mediated penetration of the rice leaf. In this review, we discuss the potential metabolic sources of glycerol in the rice blast fungus and how appressorium turgor is focused as physical force at the base of the infection cell, leading to the formation of a rigid penetration peg. We review recent studies of *M. oryzae* and other relevant appressorium-forming fungi which shed light on how glycerol is synthesised and how appressorium turgor is regulated. Finally, we provide some questions to guide avenues of future research that will be important in fully understanding the role of glycerol in rice blast disease.

Introduction

Rice blast is the most serious disease of cultivated rice, which each year destroys enough rice to feed 60 million people (Talbot, 2003). It is among the most significant global crop diseases and occurs wherever rice is grown. Rice blast is currently causing serious outbreaks in sub-Saharan Africa (Kihoro et al., 2013), as well as

across South Asia, and South America (Martin-Urdiroz et al., 2016). The rice blast fungus *Magnaporthe oryzae*, which causes the disease, gains entry to its host by developing a specialised infection structure called an appressorium, which allows direct penetration of the plant leaf surface (Ryder and Talbot, 2015; Martin-Urdiroz et al., 2016). *M. oryzae* appressoria develop enormous turgor of up to 8.0 MPa (80 bars), which is necessary for rice infection (Howard et al., 1991), Appressoria become melanin-pigmented due to a thick layer of melanin being deposited on the inner side of the appressorium cell wall. Melanin creates a semi-permeable barrier to facilitate the accumulation of solutes and subsequent generation of high turgor pressures within the cell. As a consequence, melanin biosynthetic mutants are non-pathogenic and cannot generate cellular turgor in appressoria (Chumley and Valent, 1990). Melanin provides a barrier to glycerol influx/efflux, which is demonstrated by the rapid recovery from appressorial incipient cytorrhysis of melanin deficient mutants, but not by normally melanised appressoria (de Jong et al., 1997).

The major solute that accumulates in appressoria is glycerol, which has been shown to reach concentrations in excess of 3M (de Jong et al., 1997). *M. oryzae* can form infection structures in the absence of exogenous nutrients and therefore appressorial glycerol must be derived from storage carbohydrates present in the spore from which they develop. Rice blast disease starts when a three-celled conidium lands on the rice leaf surface and germinates, sending out a short polarised germ tube (Figure 1). This hooks and flattens against the leaf surface, before elaborating the dome-shaped appressorium at its tip (Veneault-Fourrey et al., 2006). The timing of events is important; by 4 hours post-germination a septum forms, separating the appressorium from the germ tube (Saunders et al., 2010). This is linked to a single round of mitosis in the conidial cell from which the germ tube emerges, and the initiation of appressorium formation is regulated by a cell cycle checkpoint operating at S-phase (Saunders et al., 2010). Appressorial melanisation is clearly visible from 8h onwards and incipient cytorrhysis assays indicate that turgor pressure begins to be generated in the appressorium from 16 h (Figure 1) and penetration of the rice cuticle can occur from 24 h onwards.

Given the current depth of understanding of the transcriptional and proteomic fluxes that accompany disease-related development (Soanes et al., 2012; Franck et al., 2013) and the importance of *Magnaporthe oryzae* as the most serious pathogen of

rice and as a leading model cereal pathogen (Yan and Talbot, 2016; Martin-Urdiroz et al., 2016), the following discussion will address the pressing need to reconsider glycerol formation in the broader context of recent discoveries.

Potential precursors of appressorial glycerol

As appressoria of *M. oryzae* develop in dewdrops, the fungus does not take up any nutrients until it has entered the first rice epidermal cells. Appressorium development, turgor generation and penetration peg formation are therefore all fuelled by storage compounds within the three-celled conidium (see Figure 1). The potential storage reserves present in the spore include glycogen, lipid droplets, mannitol and trehalose. Each of these storage reserves has therefore been studied to determine their contribution to appressorium turgor generation.

Glycogen reserves are abundant in ungerminated conidia and rapidly consumed during germination (Bourett and Howard 1990; Thines et al., 2000). Mutants lacking the *AGL1*-encoded amyloglucosidase and the *GPH1*-encoded glycogen phosphorylase generate normal appressorial turgor and penetrate rice leaves, but cannot proliferate in rice leaf tissue (Badaruddin et al., 2013). Glycogen mobilisation therefore does not contribute significantly to appressorium turgor generation in *M. oryzae*. However, it is clear the glycogen metabolism is important in subsequent pathogenic development and likely contributes to the overall fuelling of the infection process, albeit with a more major role at a point of rapid cell wall biosynthesis by the fungus as it develops secondary invasive hyphae to colonise rice tissue.

Conidia of *M. oryzae* contain numerous lipid droplets and lipid degradation has been proposed as a major route to glycerol accumulation in appressoria (Thines et al., 2000). Consistent with this idea, high levels of triacylglycerol lipase activity have been observed in germinating spores of *M. oryzae* and the enzyme activity increases during appressorium development, concomitant with the breakdown of lipid bodies (Thines et al., 2000). There is, however, very significant functional redundancy within triacylglycerol lipase-encoding genes in *M. oryzae* (Wang et al., 2007). Deletion of any of the 8 predicted intracellular triacylglycerol lipase-encoding genes in *M. oryzae* did not prevent plant infection by the fungus, for example. This included enzymes predicted to be lipid droplet-associated lipases, patatin-like phospholipases, and orthologues of hormone-dependent lipases, as well as the major predicted cytoplasmic

triacylglycerol lipases (Wang et al., 2007). There are, however, further predicted lipase and esterase-encoding genes in the latest annotated versions of the *M. oryzae* genome sequence that have yet to be characterised. Interestingly, mutation of the *MFP1* gene, which encodes the multifunctional-β-oxidation protein causes a reduction in virulence in *M. oryzae*. This is due primarily to reduced melanisation, highlighting the need for lipid metabolism in also fuelling polyketide biosynthesis in the developing appressorium (Wang et al., 2007).

It is clear that autophagy in the *M. oryzae* conidium is an absolute requirement for appressorium function. Mutants that lack components of the autophagic machinery, such as Atg8 or the Atg1 kinase, form non-functional appressoria (Veneault-Fourrey et al., 2006) and generate less appressorial turgor (Liu et al., 2007). A comprehensive genome-wide analysis furthermore demonstrated that all components of the macroautophagy pathway are necessary for appressorium-dependent plant infection by *M. oryzae*, whereas impairing selective autophagy was dispensable for rice blast infections (Kershaw and Talbot, 2009). However, as autophagy constitutes the mass recycling of all cellular components, including organelles and cytoplasm, determining the relative contribution of different components (carbohydrates, proteins, lipids) to glycerol formation is challenging. It is known that under starvation conditions in the budding yeast *Saccharomyces cerevisiae*, and during infection-related morphogenesis in *M. oryzae,* lipid droplets are taken up by vacuoles (Moeller et al., 1979; Weber et al., 2001). In budding yeast, the vacuolar lipase Atg15 is required to lyse several different types of autophagy-destined bodies including lipid droplets when they enter the vacuole (Takeshige et al., 1992). Indeed, under certain conditions in yeast, deletion of the *ATG15* gene leads to decreased rather than increased lipid droplet content, possibly due to compensatory induction of TAGs in the cytosol (Maeda et al., 2015). Therefore, although *ATG15* is required for virulence in *M. oryzae* (Kershaw and Talbot, 2009) and in the wheat and barley pathogen *Fusarium graminearum* (Nguyen et al., 2011), it is currently difficult to disentangle the pleiotropic effects of the loss of this gene and determine a precise function that might be associated directly with appressorium glycerol generation. In summary, bulk autophagy of the contents of the three-celled conidium and their trafficking to the appressorium are essential for appressorium turgor generation, melanisation and re-polarisation. Without autophagy the fungus is rendered completely non-pathogenic.

Furthermore, it seems likely that triacylglycerol is one of the major sources of appressorial glycerol, but determining how glycerol synthesis occurs is complicated by the extensive redundancy in lipase-encoding genes in the fungus. Significant further study will therefore be necessary to define the precise contributions of individual enzymes. However, very recent results which catalogue the appressorial transcriptome and proteome (Soanes et al., 2012; Franck et al., 2013) will greatly focus future efforts and new technologies hold promise for facilitating analysis of gene families.

The enzymatic machinery for glycerol production in *Magnaporthe oryzae*

To investigate the origins of appressorial glycerol in *M. oryzae*, the major enzymatic activities associated with its synthesis and the expression patterns of the genes that encode them, have been analysed and are presented in Figure 2. Both glycerol-3-phosphate dehydrogenase (Gpd1) and glycerol dehydrogenase (Gfd1) enzyme activities have been detected in mycelium and appressoria of *M. oryzae*, although significant induction of these enzymes during infection-related development was not apparent (Thines et al., 2000). Strong transcriptional induction of *GPD1,* encoding the cytosolic form of glycerol-3-phosphate dehydrogenase, is apparent upon germination (our unpublished data). Without a similar induction in the transcription of the glycerol-3 phosphatase, this would provide a significant pool of glycerol-3-phosphate for lipid formation. There is, however, also substantial metabolic flow through to glycerol (or glycerol formation via a Gpd1-independent route) because accumulation of glycerol peaks at 8 hours post germination (De Jong et al., 1997). Accumulation of glycerol following germination has also been reported in *Aspergillus* species (d'Enfert and Fontain, 1997). In *Aspergillus nidulans* (and closely related species) turgor generation may play an important role in spore germination because conidia of this fungus swell conspicuously before germination. In *M. oryzae* the requirement for glycerol-3-phosphate must be met in the absence of significant external nutrients. Given observations so far, it seems reasonable to assume that upon germination, Gpd1-dependent formation of glycerol from dihydroxyacetone phosphate (DHAP), contributes significantly to meeting the glycerol-3-phosphate demand rather than dephosphorylation of glycerol by glycerol kinases. In *Aspergillus nidulans* glycerol formed during spore germination is the product of the conversion of dihydroxyactone (DHA) to glycerol by the glycerol dehydrogenase gldB rather than the alternative

glycerol-3-phosphate dehydrogenase (GfdA)-dependent route (de Vries et al., 2003). In *M. oryzae*, however, there is no clear induction in the activity of these enzymes in either pathway (Thines et al., 2000). Indeed, there is only the initial induction in transcription of the glycerol-3-phosphate dehydrogenase-encoding gene *GPD1* upon germination (our unpublished data). Another likely component in glycerol synthesis is the *HAD1* gene product. Based on analysis of its orthologue in *Fusarium fujikuroi*, (Garcıa-Martınez et al 2014), the *HAD1* (MGG_00099) gene product very likely dephosphorylates dihydroxyacetone phosphate (DHAP) and, together with the *GFD1* gene product (the corresponding gene is physically associated with *HAD1* in the *M. oryzae* genome), acts to produce glycerol from dihydroxyacetone-3-phosphate (see Figure 2). The likely *Neurospora crassa* orthologue of *HAD1, CUT*, is additionally involved in the response to osmotic stress (Lew and Levina, 2007). In *M. oryzae GFD1* and *HAD1* seem to share a common promoter region, and these genes are markedly, and co-ordinately, transcriptionally induced in maturing appressoria from 16 hpi onwards and also later during growth in rice tissue (our unpublished data). Their role in appressorium turgor generation and rice infection has yet to be tested.

The predicted mitochondrial form of glycerol-3-phosphate dehydrogenase (MGG_03147) is likely, by homology, to be an FAD-dependent enzyme that catalyses the reverse reaction to the cytosolic form of the enzyme (Gpd1). Together, both forms of glycerol-3-phosphate dehydrogenase also play an important role in transporting reducing equivalents across the mitochondrial membrane by a shuttle mechanism (similarly to the malate shuttle) for use in oxidative phosphorylation. Coupling the activity of the mitochondrial and cytosolic forms of glycerol-3-phosphate dehydrogenases therefore provides a means whereby NADH generated through glycolysis can be converted into energy through respiration.

In *Aspergillus nidulans*, despite the fact that glycerol is formed by the GfdA glycerol dehydrogenase-dependent pathway upon spore germination, this activity is not required for germination to occur, except under conditions of osmotic stress (de Vries et al., 2003). A double mutant lacking both glycerol-3-phosphate dehydrogenase and glycerol dehydrogenase is, however, only able to grow normally when glycerol is supplied extracellularly (de Vries et al., 2003). In yeast, glycerol accumulation under osmotic stress is produced predominantly by the NADH-dependent glycerol-3-phosphate dehydrogenase route (Blomberg and Adler, 1989; Thevelein, 1994). In

Aspergillus nidulans, however, although both pathways contribute to glycerol production, the glycerol-3-phosphate dehydrogenase-dependent pathway is the major route to glycerol under conditions of osmotic stress (Fillinger et al., 2001). There may, additionally, be compensatory production of other compatible solutes under osmotic stress when glycerol production is blocked genetically (de Vries et al 2003).

It is known that in the absence of glycerol-3-phosphate dehydrogenase activity, glycerol-3-phosphate can be formed by glycerol uptake and then conversion to glycerol-3-phosphate by the action of glycerol kinase. However, *M. oryzae* spores germinate with no external nutrient supplies and would be very unlikely to meet their glycerol-3-phosphate demand by glycerol uptake. One predicted *M. grisea* glycerol uptake protein has been identified with significant similarity to the Gut2p glycerol uptake protein of *S. cerevisiae* (Holst et al., 2000) and, given that it is transcribed constitutively throughout appressorium development (our unpublished data), this enzyme could conceivably act to recover any glycerol lost to the environment, although this has not been demonstrated experimentally. Surprisingly, in *Aspergillus nidulans* glycerol-3-phosphate can be formed by an unknown mechanism in the absence of both glycerol-3-phosphate dehydrogenase and glycerol uptake protein GlcA (Fillinger et al., 2001). An *Aspergillus nidulans* mutant lacking the glycerol-3-phosphate dehydrogenase enzyme GfdA has an approximately tenfold reduction in glycerol-3-phosphate content suggesting that, in contrast to glycerol formation, glycerol-3-phosphate formation in *A. nidulans* is primarily dependent on glycerol-3-phosphate dehydrogenase. Surprisingly, given the role of glycerol-3-phosphate as a precursor to membrane lipids, the phospholipid composition of *gfdA* mutants was not found to be affected (Fillinger et al 2001). Integrity of the cell wall at hyphal tips is affected, however, providing a potential link between glycerol metabolism and cell wall integrity.

The situation in the appressorium-forming pathogen *Colletotrichum gloeosporioides* also differs from that in *A. nidulans* because glycerol-3-phosphate dehydrogenase has been shown to be the major route to glycerol production, in a similar manner to the hyperosmotic stress response by budding yeast (Wei et al., 2004). Strains of *C. gloeosporioides* lacking glycerol-3-phosphate dehydrogenase show reduced glycerol-3-phosphate and glycerol content and are only able to sustain growth when exogenous glycerol is applied. These mutants do, however, have functional appressoria but have a reduced ability to proliferate in plant tissue (Wei et al., 2004).

In summary, *M. oryzae* possesses and expresses all of the enzymatic machinery for glycerol production by various routes, as shown in Figure 2. Some of these genes have proven difficult to isolate and characterise in *M. oryzae*, including most notably the *GPD1* gene, and it is still unclear which enzyme constitutes the principal route by which glycerol is made, or ultimately metabolised. A systematic analysis of all of the glycerol-associated genes and the enzymes they encode, will clearly be necessary to make further progress. It is clear, however, that glycerol biosynthesis in *M. oryzae* differs markedly from the well known experimental model fungi, the budding yeast *S. cerevisiae* and the filamentous ascomycete *A. nidulans*, which of course also differ markedly from one another. Glycerol metabolism is therefore divergently configured and regulated by fungi, in response to developmental cues and external stresses, respectively.

Regulation of appressorium turgor generation

In order to bring about plant infection, the *M. oryzae* appressorium must produce a rigid penetration peg at its base, which ruptures the rice leaf cuticle and epidermal cell wall. This allows the fungus entry to rice tissue. The fungus must, therefore, have a means by which is can focus appressorium turgor at the base of the cell, where it contacts the rice leaf, and must be able to re-organise its cytoskeleton in order to ensure rapid force generation and polarised fungal growth from a central specific point. Recent evidence has shown that these process are dependent on septin GTPases, which form a hetero-oligomeric ring structure around the appressorium pore– a region of the base of the appressorium that lacks melanin and has a very thin cell wall compared to the rest of the infection cell (Bourett and Howard, 1990). The septin ring acts as lateral diffusion barrier, which ensures that polarity determinants, such as Las17, which forms part of the Arp2/3 complex involved in actin polymerisation, and endocytic factors such as BAR domain proteins, are held in the right location. Critically, the septin ring also provides cortical rigidity and scaffolds F-actin in a toroidal network around the appressorium pore (Dagdas et al, 2012). Upon re-polarisation, which may itself be initiated by septin-dependent membrane curvature generation, rapid actin polymerisation occurs as the penetration hypha extends through the rice leaf surface. Interestingly, assembly of the F-actin network at the appressorium pore is regulated by the Nox2 NADPH oxidase complex (Ryder et al., 2013), suggesting that regulated synthesis of reactive oxygen species is important in controlling F-actin dynamics,

which are necessary for penetration peg development. Rapid protrusion of the penetration peg also requires polarised exocytosis at the appressorium pore, to ensure trafficking of new plasma membrane at the penetration peg tip and rapid cell wall biogenesis. The octameric exocyst complex, which is necessary for polarised protein secretion, is organised in a ring conformation at the appressorium pore which has also been shown to be a septin-dependent process (Gupta et al., 2015).

The process by which the appressorium re-polarises can only occur once maximal turgor is achieved by the cell. The appressorium must, therefore possess a mechanism to monitor turgor and modulate glycerol generation, in order to then translate turgor into force generation at the base of the cell. Regulation of cellular turgor has been best studied in micro-organisms in response to osmotic stress and in *S.cerevisiae* it is well known that the accumulation of glycerol is regulated by the HOG (High osmolarity glycerol) MAP kinase pathway, which activates glycerol production by Hog1 MAP kinase-mediated phosphorylation of the glycerol-3-phosphate dehydrogenase, Gpd1 (Albertyn et al., 1994). The *M. oryzae* orthologue of Hog1 is encoded by the *OSM1* gene (Dixon et al. 1999) and *osm1* mutants are sensitive to osmotic stress. Importantly, however, *osm1* mutants form normally functional appressoria which generate turgor. Glycerol synthesis in the appressorium is therefore HOG-independent in *M. oryzae*. Consistent with this, the major osmolyte in mycelium of *M. oryzae* exposed to osmotic stress is arabitol, which is regulated by the Osm1 MAP kinase, even when appressoria are osmotically stressed (Dixon et al., 1999). Identifying the turgor-sensing mechanism of appressoria is under active investigation currently, including the characterisation of stretch-activated gated ion channel proteins in the plasma membrane, mechanosensory proteins and other potential osmoregulatory kinases (L. Ryder and N.J. Talbot, unpublished). This is being carried out by utilising the range of mutants involved in the septin-dependent repolarisation pathway in genetic screens to identify putative turgor sensory components.

Regulation of the movement and metabolism of energy reserves, downstream of the autophagic process by which they are generated, appears to involve the cyclic cAMP-dependent protein kinase A pathway and Pmk1 MAP kinase (Thines et al., 2000). The cAMP-dependent protein kinase A-encoding gene *CPKA* of *M. oryzae* is required for appressorium maturation and virulence. Mutants lacking this gene show delayed, delayed mobilisation of glycogen and lipid bodies and reduced turgor generation

(Thines et al., 2000) and although mutants lacking Pmk1 do not form appressoria the mobilisation of glycogen and lipid reserves from the conidium is negatively affected. Integrating the role of these signalling pathways, with appressorium turgor sensing mechanisms is a key priority for future research and recent global transcriptional and proteomic approaches have laid a powerful framework for addressing regulation by these factors (Soanes et al., 2012; Franck et al., 2013).

Future research directions

In spite of considerable progress in understanding how appressoria both develop and function, there is still much that is poorly understood. The precise mechanisms by which glycerol is generated in the appressorium have proven elusive to identify. This is largely a consequence of only being able to generate single gene mutations in *M. oryzae*, coupled with the difficulty of carrying out biochemical analysis on appressoria, which are small cells (8 μm in diameter) that can only be induced to form in relatively small numbers on hydrophobic surfaces. Generating sufficient biomass of appressoria for metabolite analysis and labelling studies, has not yet been possible. However, it is clear that new, highly sensitive mass spectrometry methods should yield results from tiny amounts of starting material, and this should allow more detailed temporal analysis of metabolite concentrations to proceed, with associated enzymatic assays. The other very exciting development is the potential of CRISPR-Cas9 editing to allow whole gene families to be mutated simultaneously, or in specific combinations. This should allow a much clearer dissection of the role of the lipase family, for instance, in appressorium turgor generation, and the role of the major glycerol biosynthetic enzymes. When carried out in combination with RNA-seq analysis, which is already well established for appressorium preparations, this should provide a more integrated analysis of the control of glycerol synthesis and turgor control.

There are also interesting questions regarding the spatial control of glycerol synthesis that also need to be addressed. The vacuole of *M. oryzae* appressoria expands during the formation of the cell and nearly fills the cell at maturity (Weber et al., 2001). What drives the expansion of the vacuole is unclear at present and the mechanism and the role of this expansion, are interesting questions for future research. As glycerol could only be compartmentalised at very significantly energetic cost to the fungus it would seem likely that other solutes, which might be detrimental to normal enzymatic

activities in the cytosol, could accumulate in the vacuole. It will be interesting to determine whether the active uptake of cations may allow vacuolar expansion by osmotic action while at the same time balancing the charges generated by the production of superoxide by the NADPH oxidase Nox2 present in the vacuolar membrane (see review by Segal, 2016). Interestingly, several of the predicted cation transporter encoding genes in *M. oryzae* show dramatic transcriptional activation in maturing appressoria and, given indications for the role of ions in turgor regulation in plants and fungi (Shabala and Lew, 2002; Lew et al., 2006), it would be of interest to explore the subcellular localisation and function of members of this gene family. Another question concerns the need to maintain the redox balance of the appressorium. Whether the NADH-consuming glycerol-3-phosphate dehydrogenase route might be favoured over the NADPH-consuming glycerol dehydrogenase pathway to glycerol may, for example, depend on the relative rates of flux through glycolysis (NADH producing) and gluconeogenesis, and additionally on the contribution of the pentose phosphate pathway to the biosynthetic activities of the appressorium during plant infection. Only detailed metabolomic analysis, as described above, coupled with molecular genetic characterisation of all associated genes, and labelling experiments, will be able to address this question.

Glycerol at molar concentrations also has chaotropic effects which may be balanced by concomitant accumulation of kosmotropic metabolites or ions (de Lima Alves et al., 2015). Spores of *Magnaporthe* are able to germinate in 2M glycerol solutions, however their development is significantly delayed (our unpublished data). Interestingly, recent transcriptome analysis has indicated that the transcription of a predicted mannitol-1-phosphate 5-dehydrogenase, is strongly induced at a time point when turgor would be expected to be maximal (there is a nearly 8 fold higher transcript abundance at 24h - only after this time point is penetration normally observed - compared to at 6 h). Therefore, it may be that mannitol accumulates as an accessory compatible solute in order to modulate the chaotropic effects of molar concentrations of glycerol. Interestingly mannitol has also recently be shown to accumulate during later stages of infection of rice plants concomitantly with glycerol accumulation (Parker et al., 2009) and and, given its kosmotropic effects (Cray et al., 2013), mannitol might therefore also be acting to balance the chaotropic effects of glycerol at this stage too. Addressing

such issues is important and will be greatly assisted by new gene editing technologies which will enable multiple mutations to be created in one strain.

It will also be of interest to address the broader functions of glycerol outside of appressorial turgor generation. Glycerol has also been found to accumulate at later stages of infection of rice by *M. oryzae* (Parker et al., 2009). The function of glycerol at this stage may be to allow the fungus to grow even under conditions of low water availability as would be anticipated in heavily infected leaves which become desiccated. A function for glycerol in the ability of propagules of xerophilic fungi to germinate under extremes of water availability has recently been established (Stevenson et al., 2016). Heavily infected plants might also be prone to competition for resources by other fungi which attempt to 'piggyback' the infection by Magnaporthe. Glycerol accumulated within infectious hyphae of the rice blast fungus might also provide a competitive advantage over other fungi not so well adapted to the dehydrated environment encountered at late stages of infection.

In summary, glycerol plays an absolutely critical role in the function of the appressorium of the rice blast fungus *M. oryzae.* It is responsible– as the major compatible solute in appressoria –for generating an extraordinarily high pressure of up to 8.0MPa. We are beginning to understand how cellular turgor in the appressorium results in re-polarisation of the cell and penetration hypha development for plant infection, but there is still much to learn about how glycerol is made, where it is made, and how the process is genetically controlled. Fortunately, many of the tools needed to address these questions are now becoming available, so it promises to be an exciting time ahead.

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Figure Legends

Figure 1. A. Infection-related development by the rice blast fungus *Magnaporthe oryzae.* A three celled pyriform conidium germinates and elaborates a dome-shaped single-celled appressorium at the germ tube tip. The appressorium becomes darkly pigmented due to deposition of a layer of melanin in the cell wall, which is essential for turgor generation and plant infection. Appressoria were allowed to form on hydrophobic cover slips and photographed using brightfield illumination at times shown (Bar = 10 μm). **B**. An incipient cytorrhysis assay was used to determine the cellular turgor of appressoria during development. Appressoria were formed as in **A**.

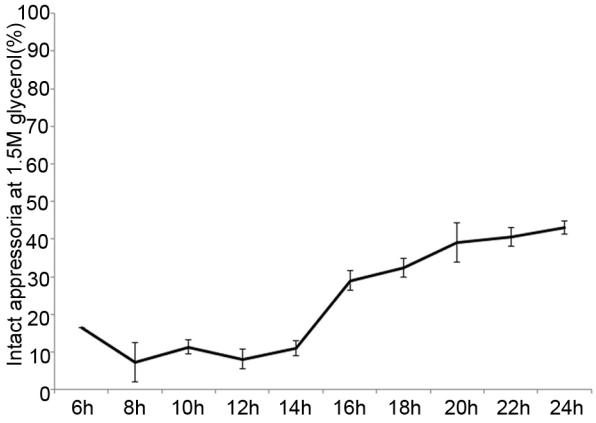
and extracellular glycerol added to a final concentration of 1.5 M. The proportion of intact cells following glycerol exposure was measured, as a means of estimating appressorial turgor and intracellular solute concentration.

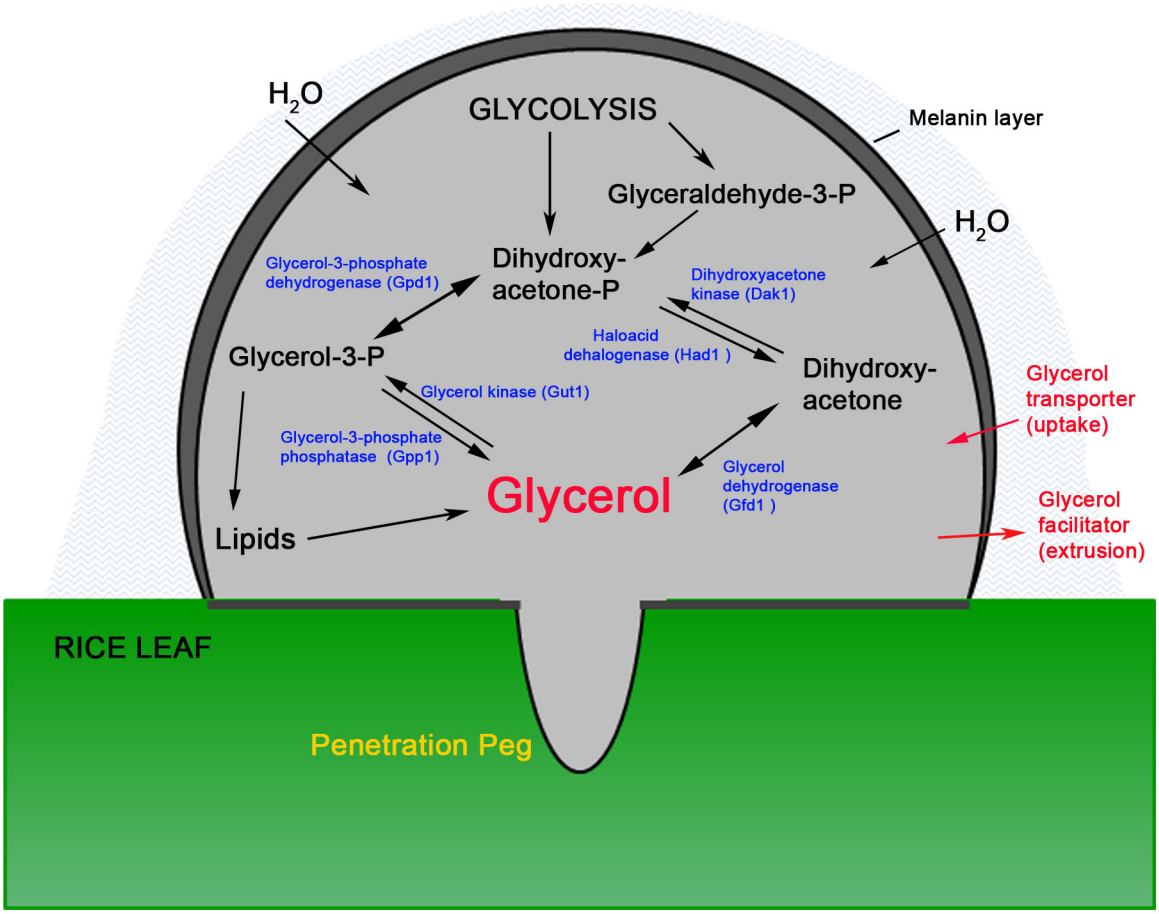
Figure 2. Potential metabolic routes for glycerol generation, together with corresponding enzymes predicted to be present in the *M. oryzae* appressorium. The appressorium generates turgor to facilitate rupture of the rice cuticle by a rigid penetration peg, as shown. Glycerol can be generated from lipids via the extensive triacylglycerol lipase activities detected in appressoria. In addition, there are two alternative pathways for glycerol synthesis from glycolysis that are present in *M. oryzae*, as shown. Gene names used in this review and corresponding enzymes are shown in blue. An additional enzyme activity likely to be present, but not shown, is the mitochrondrial glycerol-3-phosphate dehydrogenase which forms dihydroxyacetone phosphate by oxidation of glycerol-3-phosphate and which normally acts to maintain redox balance.

A


4h
6h
8h
12h
16h
24h

B


Intact appressoria at 1.5M glycerol(%)
100
90
80
70
60
50
40
30
20
10
0
6h
8h
10h
12h
14h
16h
18h
20h
22h
24h


H_2O
GLYCOLYSIS
Melanin layer
Glyceraldehyde-3-P
H_2O
Glycerol-3-phosphate dehydrogenase (Gpd1)
Dihydroxy-acetone-P
Dihydroxyacetone kinase (Dak1)
Haloacid dehalogenase (Had1)
Glycerol-3-P
Dihydroxy-acetone
Glycerol kinase (Gut1)
Glycerol transporter (uptake)
Glycerol-3-phosphate phosphatase (Gpp1)
Glycerol
Glycerol dehydrogenase (Gfd1)
Glycerol facilitator (extrusion)
Lipids
RICE LEAF
Penetration Peg